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Patentanmeldung Nr.
Patent application no.
Demande de brevet n° PCT/EP 02/14877

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: PCT/EP 02/14877
Demande n°:

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Sheet No. ...3...

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PCT/EP 02/14877

A VERSATILE *IN VITRO* SYSTEM TO DETECT HORMONAL ACTIVITY OF SINGLE COMPOUNDS, MIXTURES, ENVIRONMENTAL- AND BIOLOGICAL SAMPLES

BACKGROUND

Identification of biologically active compounds is important in a variety of fields, ranging from pharmacological- and clinical screening, food and feed safety, toxicological monitoring and risk assessment. Traditionally, monitoring strategies focus on usage of two extremes: sophisticated, detailed chemical analysis, and determination of biological effects using whole animal assays, and epidemiology. With those methods a correlation can be made between internal or environmental levels of a chemical and the effect seen in an organism (exposure and effect determinations). Major problems occur when chemical analysis is inadequate to predict biological effects because of metabolism of the parent compound to metabolites with unknown biological activity, or when complex mixtures of biologically active compounds are present, which is always the case in environmental- and food matrices. Also the biological effect determination in experimental animals has various drawbacks, in particularly with respect to capacity, costs, ethics, speed and relevance to humans. With many new chemicals, food additives and pharmaceuticals entering the marked each year, high throughput testing strategies have to be developed. The revolution in molecular biology and biotechnology has allowed development of a broad spectrum of novel high speed testing strategies using cultured cells, isolated biological endpoints and genetically modified organisms. Rapid advance in automated screening technologies and bioinformatics create additional possibilities to replace existing whole animal and chemical assays for biological active compounds by new mechanism-based biodetection systems. In particular, in a tiered approach these in vitro detection systems are ideal for first line screening (Tier 1), directing further decisions on more extensive hazard identification using animals in the case of effect assessment of known compounds or compound identification using chemical analysis in the case of quality control of environmental- and food, feed or blood samples.

Biological endpoints that have priority in hazard identification of chemicals are carcinogenicity, mutagenicity and reproductive toxicity ('CMR' substances; EU White Paper, Strategy for a future chemicals policy) or POP (persistent organic pollutants) characteristics. In reproductive toxicity emphasis has currently been laid on chemical interfering with the nuclear hormone receptor systems activated by androgens, estrogens and thyroid hormones.

Steroid hormones are essential in most reproductive processes and can influence many other physiological processes as well. Due to the relatively simple chemical structure and lipophylic nature of steroids, their regulatory pathways can be easily modified by pharmacological-, environmental- and dietary agents. Because of this, steroids and steroid-mimicking compounds find applications in many fields and their detection is

important in doping control, meat quality control, medical practice, environmental- and food and feed monitoring, etc.

Steroid hormones are nuclear hormone receptor ligands that enter cells by diffusion where they bind to receptors. Five major types of steroid receptors are known; those for estrogens, androgens, progestins, glucocorticoids and mineralocorticoids. Upon ligand binding these receptors become activated, and they will enter the nucleus and bind to recognition sequences in promoter regions of target genes, the hormone responsive element. The DNA bound receptor will activate transcription of the target gene. This will lead to new protein synthesis and an altered cellular functioning.

We have developed a simple screening assay for estrogenic compounds making use of the fact that the receptor for estrogens is a transcription factor that induces transcription of target genes after binding to specific DNA sequences in their promoter (Legler et al., 1999; see figure 1). When these DNA sequences are linked to the gene of an easily measurable protein (the so-called "reporter gene") and stably introduced in a suitable cell line, an estrogen responsive reporter cell line is generated allowing large scale screening of chemicals. We generated a highly selective and responsive reporter gene with exceptionally low background activity in which three estrogen responsive elements were coupled to a very minimal promoter and luciferase. Upon stable introduction in human T47D breast carcinoma cells a highly sensitive biological detection system was generated, the ER-CALUX assay (Legler et al., 1999).

Accordingly, we developed a versatile system that allows rapid in vitro screening on many prioritary effects. To this end we have generated a range of stably transfected cell lines with specific receptors and suitable highly selective reporter genes in which we can detect ligands activating a wide variety of biologically relevant endpoints, making them particularly suitable to detect biological activity of single compounds, mixtures (e.g. present in food and feed), environmental samples and biological samples (e.g. meat, serum, plant and animal homogenates). Our system allows measurement of activity of compounds, regardless of their chemical nature. An advantage of our approach is that it is simple, rapid and animal friendly, while still covering an array of endpoints that may be targeted by chemicals, or natural compounds. It is highly sensitive, and is also suitable in quality control procedures since it will also detect effects of (intentionally) masked compounds or complex mixtures thereof, that now escape chemical detection. Due to a method that we called 'effect profiling' a preliminary identification of compounds can be made which will be followed by chemical identification, when necessary.

SUMMARY OF THE INVENTION

According to one aspect this invention concerns an assay for determining beneficial or harmful effects of a sample, based on the spectrum of biological activities of said sample, with the following components:

1. Contacting the sample in parallel with a battery (more than three) of cell lines, preferably from the same parent cell line, in particular human osteoblastic cell lines like U2-OS, all stably expressing a reporter gene product that responds to a specific cellular signaling pathway (e.g. steroid- or cytokine activated pathways, DNA damage). In addition to the reporter gene one or two expression plasmids

coding for a specific rate-limiting component (e.g. a receptor) of the specific signaling route is expressed if the said cell line is deficient of such a factor. It may also concern a gene coding for a ligand modifying factor, such as a metabolic enzyme. The means to induce the expression of the said gene for an additional (rate limiting or modifying) component may also be by pharmacological activation leading to expression of genes endogenously contained in the genome of the reporter cell.

2. Determine the activity of the sample towards the different cellular endpoints by measuring reporter gene (e.g. luciferase) activity in the individual cell lines.
3. Analysis of the results by comparing the activities in the different cell lines. This will show a profile of biological activities that can be used to make predictions on the expected biological activity of the said sample in a broader sense, e.g. its expected toxicological-, pharmacological- or nutritional properties. This system, which we called 'effect profiling', can also be used as a first step in the chemical identification of the biological active components if the said sample consists of an unknown mixture of components. This can be done by comparing the effect profile identified with profiles determined with samples of known composition.

According to another aspect this invention concerns human osteoblastic cell lines, particularly the U2-OS cell line, as versatile recipients to generate reporter cell lines. In particular the U2-OS cell line was found to be suitable to generate a range of highly specific and responsive reporter gene assays, which makes it very suitable to carry out the effect profiling described above.

The invention also concerns a method for identification of the suitable human osteoblastic cell lines, particularly the U2-OS cell line, to generate highly responsive reporter gene assays for nuclear hormone receptor ligands, such as those activating the androgen-, progesterone-, glucocorticoid-, estrogen (alpha and beta)-, and thyroid receptors. The extreme responsiveness of the receptors in these cells, which was particularly evident for the steroid receptors make these cells not only suitable for screening in an 'effect profiling' setting but also as individual cell lines, to measure the specific endpoints addressed with the expressed reporter gene when. The invention also relates to such reporter cell lines expressing additional rate limiting or modifying components to modulate response, as described above.

The invention also relates to U2-OS cells which express multiple components for additional signaling routes involved in biological responses to prioritary endpoints of toxicological and pharmacological concern, such as apoptosis, cell death, metal responses, cytokine-, stress-, DNA damage- and growth factor inducible transcriptional responses (NF-kappaB, AP-1, STAT, p53), retinoid- and dioxin receptor inducible pathways. Specific reporter gene assays measuring activation of such pathways in the said cells are also covered by the invention.

Yet another aspect of the invention concerns alternative methods to measure protein and mRNA expression, e.g using DNA arrays, using human osteoblastic cell lines such as U2-OS, with the aim to detect biological activity of single compounds, mixtures (e.g. present in food and feed), environmental samples and biological samples (e.g. meat, serum, plant and animal homogenates).

FIGURES

Figure 1. Principle of a reporter cell line; the ER-CALUX assay.
Upon estrogen binding the estrogen receptor (ER) becomes activated, and binds to recognition sequences in promoter regions of target genes, the so-called estrogen responsive elements (EREs). Three of these EREs have been linked to a minimal promoter element (the TATA box) and the gene of an easily measurable protein (in this case luciferase). The thus obtained reporter gene was stably introduced in T47D cells. In this way the ligand-activated receptor will activate luciferase transcription, and the transcribed luciferase protein will emit light when a suitable substrate is added. The signal will dose-dependently increase as a result of increasing concentrations of ligand.

Figure 2. **Responsiveness of the U2-OS based CALUX cell lines. A. AR-CALUX, B. GR-CALUX, C. PR-CALUX, D. ERalpha/ERbeta-CALUX.** Cells were plated in 96 well plates and treated for 24 hours with hormone in culture medium containing 5% charcoal-stripped serum. Each point represents the mean of three independent experiments ± SEM.

Figure 3. Steroid hormones and their biosynthetic pathways.
Steroid hormones are generated through a metabolic pathway in which small molecular changes are made in each enzymatic conversion. Precursor molecules for one receptor type are specific hormones for other receptors (e.g. progesterone, testosterone).

EXAMPLE

General approach

To generate highly selective and responsive reporter genes with exceptionally low background activity we used the same approach as taken for the construct used in ER-CALUX cells. Reporter constructs were made using specific synthetic, multimerized responsive elements which were cloned upstream of the synthetic TATA box in the pGL3-tata-Luc vector. Stable transfectants were made, when necessary co-transfected with a specific receptor containing expression plasmid (pSG5).

The example refers to the tables given below

Cell line	ER	AR	PR	GR
T47D	•	–	•	–
HEK293	•	–	+	•
U2-OS	•	••	•	••

Table 1. Suitability of U2-OS cells to generate reporter cell lines for steroid hormone receptors using TATA-Luc reporters
Suitability of partent cells to generate additional lines. Unsuitable (–) because of failure to get receptors expressed of interfering receptors (in case of T47D cells). Suitable (+), as determined in transient transfections, or suitable as demonstrated also in stable transfcotants (•). (••) Exceptionally responsive cell lines (low EC50).

Strain	[illegible] CALUX	[illegible] CALUX	[illegible] CALUX	[illegible] CALUX	[illegible] CALUX

	precursors				
cholesterol	>-4.0				
pregnenolone	-4.5				
OH-pregnenolone	-6.8				
OH-progestrone					
androstenediol					
	progestins				
progesterone	>-4.0				
Org2058 (synthetic)					
	adrenal androgens				
DHEA	-7.6	>-4.0			
androstenedione	>-4.0				
	androgens				
testosterone	-4.1				
Dihydro-testosterone	-7.4				
	estrogens				
estrone					
estradiol					
estriol					
	corticosteroids				
desoxycortisol					
cortisol					
cortison					
Dexamethasone (synthetic)	>-4.0				
	mineralocorticoids				
desoxycorticosteron					
corticosteron					
aldosteron					
	thyroid hormones				
T4					
T3					

Table 2. Preliminary data on the potency (logEC50) of defined, natural- and specific synthetic ligands in various CALUX assays.
Red boxes; specific ligands; EC50 $\leq 10^{-9}$M. Yellow=relatively strong ligands; EC50 $\leq 10^{-7}$M $\geq 10^{-9}$M

RESULTS

We discovered that the U2-OS cell line is particularly suitable to generate reporter gene assays because of the following reasons:

1. It supported the signaling pathways of many important biological molecules. This was for instance true for steroid responses, in which it was found to be superior compared to two other cell lines (table 1). No other line is known to us that makes such highly responsive lines for a whole range of receptors, including some of the 'difficult' ones like the androgen receptor. The currently used T47D cell line in the ER-CALUX is a PR overexpressing cell, causing considerable bias towards PR mediated effects, including transcriptional interference with the other steroid receptor pathways. The HEK293 was also tested, but it had the drawback of a lower activity of GR (in terms of EC50 values) and negative results in attempts to generate a stable 293-based AR-CALUX.
2. Efficient stable transfection is possible, allowing easy expansion of the range of reporter cell lines.

3. The cells are robust, and withstand routine handling in non-specialized laboratories. Generation time is low and rapid propagation is possible in conventional serum-containing media.
4. Cells attach readily to standard cell culture materials
5. All stable cell lines remained stably transfected during more than 20 passages.

DNA constructs

Since the androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR) recognize the same DNA sequence, the same reporter gene was used for this class of receptors. The reporter construct 3xHRE-tata-Luc was constructed as followed: 3 tandem repeats of the hormone responsive element (HRE) oligonucleotides:
(AAGCTTAGAACAGTTTGTAACGAGCTCGTTACAAACTGTTCTAGCTCGTT ACAAACTGTTCTAAGCTCAAGCTT) upstream of the minimal adenovirus E1B TATA promoter sequence (GGGTATATAAT) were inserted in the multiple cloning site of the luciferase reporter construct pGL3.

The 3xERE-tata-Luc construct consisted of tandem repeats of the ERE upstream of the minimal adenovirus E1B TATA promoter sequence (GGGTATATAAT) inserted in the multiple cloning site of the luciferase reporter construct pGL3, and has been described before (Legler et al 1999).

cDNAs coding for the different receptors were introduced in the pSG5 expression plasmid.

AR-CALUX

The AR-CALUX assay was generated by stably transfecting the human U2OS cell line with pSG5-hAR and the 3xHRE-tata-Luc reporter construct. This stable cell line was characterized by its response to different steroids, as well as its non-responsiveness to other nuclear hormone receptor ligands. In contrast to other existing androgen reporter gene assays (Blankvoort et al., 2001; Vinggaard et al., 1999; Wilson et al., 2002; Terouanne et al., 2000), the AR-CALUX assay proved to excel in terms of cell line maintenance, the inducibility of luciferase activity, minimal detection limit and androgen specificity. Fig. 2A shows the responsiveness of this cell line.

GR-CALUX

The GR-CALUX assay was generated by stably transfecting the human U2OS cell line with pSG5-hGR and the 3xHRE-tata-Luc reporter construct. This stable cell line was characterized by its response to different steroids, as well as its non-responsiveness to other nuclear hormone receptor ligands. Compared to another 293-based cell line developed in our laboratories, this cell line had a 10-fold lower EC50 value. We have used this cell line to measure endogenous corticosteroid activity levels in human blood samples and have shown that it is superior to the 293 based reporter cell line and can be used to readily measure corticosteroid and synthetic glucocorticoid activity in serum. This assay can be carried out by adding serum directly to the cells. This type of application opens many possibilities, and can also be applied to other CALUX assays to measure hormonal activities of interest, e.g. total levels of estrogenic activity, which may be linked to growth of children, or risk to certain hormone related diseases. Fig. 2Bshows the responsiveness of this cell line.

PR-CALUX

The PR-CALUX assay was generated by stably transfecting the human U2OS cell line with pSG5-hPRB and the 3xHRE-tata-Luc reporter construct. This stable cell line was characterized by its response to different steroids, as well as its non-responsiveness to other nuclear hormone receptor ligands. Compared to another existing androgen reporter gene assay in hamster cells (Schoonen et al, 1998), the PR-CALUX assay proved to excel in terms of the inducibility of luciferase activity. Moreover, this is the first human assay system. Fig. 2C shows the responsiveness of this cell line.

ERalpha-CALUX, ERbeta-CALUX
We have previously generated two types of double transfectants: 1. human ERbeta with the 3xERE-tata-Luc construct. 2. human ERalpha with the 3xERE-tata-Luc construct (Queadacker et al., 2001, Schreurs et al., 2002). These lines are as responsive as the highly responsive 293-based lines (Lemmen et al., 2002), and have the additional advantage of a much better attachment to cell culture plated, making them much more easy to handle and suitable for high-throughput screening. They are essential in our effect profiling system, having the same cellular background as the above mentioned reporter cell lines. Fig. 2D shows the responsiveness of these cell lines.

Effect profiling
In our experience the U2-OS cell line is an exceptionally good line to serve in a system which we refer to as 'effect profiling'. In this system we include reporter cell lines for a broad set of biological endpoints, e.g. all steroid receptor signaling pathways. The profile of activities of a sample measured in the different cell lines gives more information on the biological risk or benefit, the specificity of the response, and the nature of the biological active components in mixture, as compared with measurement of a single endpoint.

For example, metabolism of steroids can substantially alter their biological properties, and straight-forward enzymatic conversions can lead to steroids with a totally different activity profile (see Fig.3). This can be exploited in several ways. For example, high concentrations of non-prohibited or difficult to trace precursor steroids are used in sport doping, rather than the active hormone itself. When the precursor is converted into the active hormone at the level of the target cell, as often is the case, a mechanism of detection (either chemical of biological) directed towards the active circulating hormone will be ineffective. For instance, an androgen assay that does not detect these precursors may give a high level of false-negatives. There are different ways to detect the precursor steroids in the reporter gene assay we have developed, of which the most straight forward method is the one we refer to as effect profiling. The AR-CALUX reporter line readily measures the weakly androgenic androgen precursor androstenedione (Table 2), but not DHEA. This latter steroid, however, is relatively potent in the ER-CALUX, while androstenedione is not (see table 1). The use of DHEA in our system will therefore lead to positive results in the ER-CALUX system. Even when using a relatively small panel of lines, it can be expected that all steroids test positive in one or more lines, thereby minimizing the occurrence of false-negative results. The pattern of activation already gives an indication of what type of steroid might be present in the sample. When high concentrations of endogenous estrogens are present, like is usually the case in females during their reproductive life, this minimal system will not be very suitable for the application described. However, the

discriminative power will be greatly enhanced by inclusion of more lines (measuring additional endpoints) in the system.

An additional advantage of using the expression profiling system is that if a compound gives a generalized toxic response this will show up as a repression of all reporter gene assays, irrespective of the endpoint used. More specific toxic pathways can be identified by examining patterns of responses towards various reporter gene systems. In the example described above of the androgen precursors being active in one, but not other steroid receptor systems, it will be clear that this might be caused by not only cross reaction with certain hormone receptors, but also through specific conversion to a more potent steroid for one, but not the other receptor system. When data from several defined compounds are known, specific biosynthetic routes can be deduced. In this way the biosynthetic routes can be determined in the cells, which can be known pathways, or novel, previously unrecognized ones. Clearly, though experience with a great number of endpoints and experimental samples the pattern recognition will be improved, leading to further discriminative power of the system.

Another example is the observation that cellular activation signals leading to a variety of vary different responses, such as proliferation, apoptosis, DNA repair, and differentiation are activated through signaling pathways that can have much overlap (such as p53 activation, AP1 activation, etc.). Only analysis of various signaling pathways at the same time will reveal patterns that will give information on the ultimate cellular fate , and the relevance to other cellular systems.

Additional CALUX systems

To enhance the discriminative power, and the biological relevance of the effect profiling system, multiple endpoints are measured. Fortunately, U2-OS cells express multiple components for additional signaling routes involved in biological responses to prioritary endpoints of toxicological and pharmacological concern, such as apoptosis, cell death, metal responses, cytokine-, stress-, DNA damage- and growth factor inducible transcriptional responses (NF-kappaB, AP-1, STAT, p53), retinoid- and dioxin receptor inducible pathways. It is possible to device a system in which all of these pathways are measured using reporter gene assays and/or other means to measure cellular changes (e.g., determination of protein content, mitochondrial activity, and microsomal radical formation), some of which can be combined with luciferase measurement. When combined with specific metabolic steps (see below), the current system will be applicable in a large number of fields, including screening of new and existing chemicals, environmental-, food-, feed-, sports doping-monitoring, medical research and drug discovery.

Metabolism

An additional way to improve the discriminative power of the system is to use endogenous capacity to convert steroids. For example, we have found that the 3beta HSD enzyme of which the activity includes conversion of DHEA in androstenedione is inducible in the AR-CALUX line, thereby enhancing the range of active compounds and discriminative power of the assay. An alternative way of introduction of metabolic capacity is the stable transfection of the gene coding for the enzyme or using cocultures with cells that express this enzyme, or (partially) purified enzymes.

In vivo metabolism of compounds is important to their ultimate biological activity. General metabolic steps, e.g. by liver enzymes, can be added by coculturing

appropriate cells expressing specific metabolic functions, adding cell fractions or purified enzymes.

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CLAIM

1. AN *IN VITRO* SYSTEM TO DETECT HORMONAL ACTIVITY OF SINGLE COMPOUNDS, MIXTURES, ENVIRONMENTAL- AND BIOLOGICAL SAMPLES AS DESCRIBED IN THE DESCRIPTION.

ABSTRACT

An in vitro system to detect hormonal activity of single compounds, mixtures, environmental and biological samples which system comprises determining beneficial or harmful effects of a sample, based on the spectrum of biological activities of said sample.

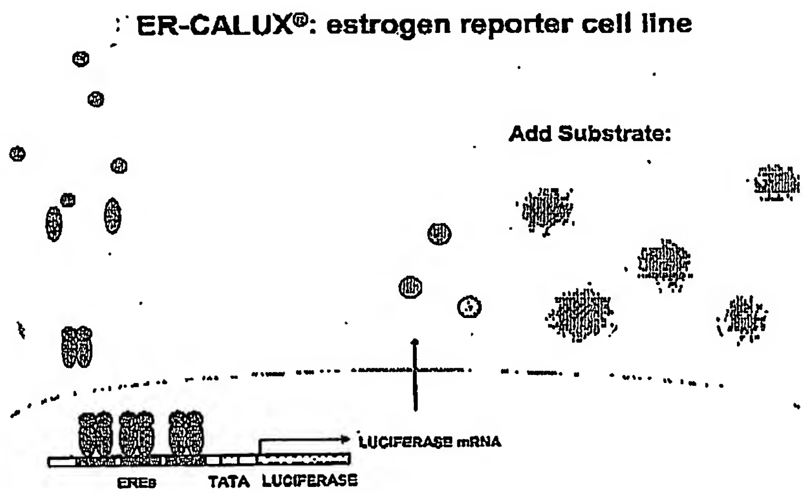
ER-CALUX®: estrogen reporter cell line
Add Substrate:
LUCIFERASE mRNA
EREs
TATA
LUCIFERASE

Figure 1

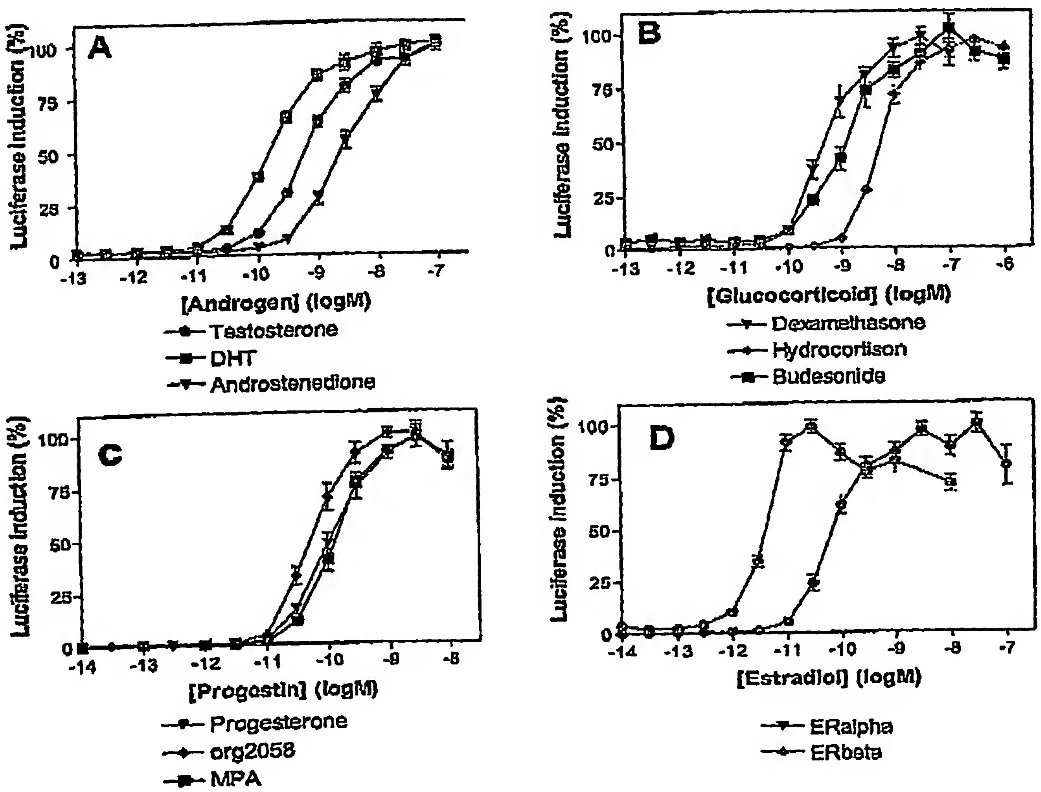
A
Luciferase Induction (%)
[Androgen] (logM)
Testosterone
DHT
Androstenedione
B
Luciferase Induction (%)
[Glucocorticoid] (logM)
Dexamethasone
Hydrocortison
Budesonide
C
Luciferase Induction (%)
[Progestin] (logM)
Progesterone
org2058
MPA
D
Luciferase Induction (%)
[Estradiol] (logM)
ERalpha
ERbeta

Figure 2

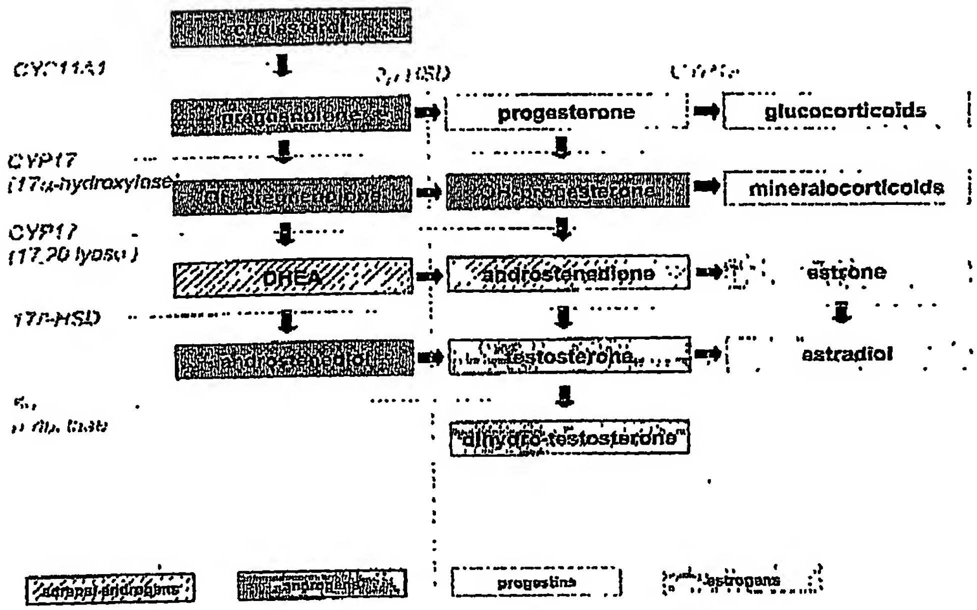
cholesterol
CYP11A1
pregnenolone
progesterone
glucocorticoids
CYP17
(17α-hydroxylase)
mineralocorticoids
CYP17
(17,20 lyase)
DHEA
androstenedione
estrone
17β-HSD
androstenediol
testosterone
astradiol
dihydro-testosterone
adrenal androgens
progestins
estrogens

Figure 3

PCT Application
PCT/EP2003/014813